Deciphering the Clinical Significance of *BRCA* Variants

A Senior Honors Thesis

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Abstract

The identification of genes predisposing individuals for specific diseases has increased the value of genetic testing. Two genes, *BRCA1* and *BRCA2*, have been shown to be significant in hereditary breast and ovarian cancers. Unfortunately, the mutation results of *BRCA* genetic testing are sometimes unclear. A class of mutations, termed "variants of uncertain significance" (VUS), provides inconclusive and unhelpful results in genetic testing because it is unknown whether these variants are cancer causing mutations or neutral polymorphisms. In our study, we combined independent variables including tumor loss of heterozygosity, co-occurrence with a known deleterious mutation *in trans*, sequence conservation or splice site analysis, pathological data (estrogen receptor, progesterone receptor, and Her2Neu status; tumor grade; and tumor histology), and personal cancer history (age of onset and cancer type) to try to classify VUS as deleterious or neutral. This selection of data allows one to assess the pathogenicity of VUS without segregation analysis or familial information, which is often unavailable because of family absence or unwillingness to undergo genetic testing. With these independent data sources, we used a modified multifactorial approach for each variant to calculate a final likelihood score. We were able to utilize our method with 98% sensitivity and 76% specificity on 57 tumor samples from 44 known deleterious variants. Additionally, we evaluated 56 tumor samples from 54 unique classified and unclassified variants. Among the 33 unclassified VUS, we quantified 21 as neutral. The classification of VUS as deleterious or neutral will aid patient care, specifically improving future decision making regarding screening, chemoprevention, prophylactic treatment, and familial risk.

Introduction

Two tumor suppressor genes, *BRCA1* and *BRCA2*, are located on chromosome 17 and 13, respectively. They encode large proteins, which function similarly in DNA damage repair to maintain the integrity of the human genome. Unfortunately, when *BRCA1* and *BRCA2* are mutated, they predispose individuals to a multitude of cancers[1]. In fact, women with *BRCA1* or *BRCA2* gene mutations are three to seven times more likely to develop breast cancer than women without mutated genes[2]. *BRCA1* and *2* are also significant in hereditary breast and ovarian cancers as the majority (~84%) of hereditary breast and ovarian cancers result from inherited mutations in *BRCA1* and *BRCA2*[3].

The high prevalence of *BRCA* mutations in hereditary cancers has made genetic testing an increasingly powerful tool as more than 70,000 individuals worldwide have undergone genetic analysis of *BRCA1* and *BRCA2* to assess their risk for hereditary breast and ovarian cancer[4]. The number of individuals undergoing testing will continue to grow as clinical genetic analysis is increasingly used for diagnosis confirmation and determining prognosis, treatment options, and future risk. Also, technological advances have made testing more accurate and economical.

The current drawback of genetic testing on *BRCA1* and *BRCA2*, however, is that approximately half, over 1500, of the unique *BRCA1* and *BRCA2* variants detected are "variants of uncertain significance" (VUS)[5]. These VUS are predominantly missense mutations or splice site mutations with unclear biological importance. Because their effect on protein function is not clear, it is not yet known whether these mutations are deleterious or neutral polymorphic changes. Deleterious changes increase cancer risk dramatically, whereas neutral polymorphic changes do not seem to increase cancer risk. Thus, individuals with genetic testing results of

VUS are given unhelpful and ambiguous data, whereas results of known deleterious mutations or known neutral mutations can appropriately guide potential screening, chemoprevention, or prophylactic surgery for patients and families.

Because many VUS are only reported in one or two individuals and many individuals do not have access to their family history, we have developed a model to assess the clinical significance of *BRCA1* and *BRCA2* VUS independent of familial information. Specifically, we used tumor loss of heterozygosity, co-occurrence with a known deleterious mutation *in trans*, sequence conservation or splice site analysis, pathological data (estrogen receptor, progesterone receptor, and Her2Neu status; tumor grade; and tumor histology), and personal cancer history (age of onset and cancer type) to try to classify VUS as deleterious or neutral.

Using these tools to analyze VUS, we developed four *BRCA* VUS classification models to assess 57 tumors with known deleterious *BRCA* mutations and 56 tumors with known neutral or unclassified *BRCA* variants. Four models were needed because *BRCA1* and *BRCA2* have different pathological characteristics, as do *BRCA* breast and ovarian cancers.

Materials and Methods

Human Samples

The proposed studies were approved by local institutional review boards, and all participants signed informed consent forms. Paired normal and tumor tissue samples were collected through one of three sources: The Ohio State University Comprehensive Cancer Center (CCC) Clinical Cancer Genetics program, Fergus Couch at Mayo Clinic, or The University of California, San Francisco (UCSF) Familial Risk Shared Resource. Inclusion into the study required a diagnosis of breast or ovarian cancer, source of normal and tumor DNA, and a *BRCA* testing report documenting a *BRCA1* and/or *BRCA2* known deleterious mutation, known neutral mutation, or VUS. A pathologist analyzed hematoxylin and eosin (H&E) stained tumor sections, and areas of the section containing >70% tumor cells were micro dissected from 10μM sections or were cored (1.6 or 2.0 mm). Normal DNA was isolated from blood, histologically normal breast tissue from tumor margins, or samples without tumor contamination. Lymphoblastoid cell lines were established from a subset of participants, also.

Genomic DNA was isolated from tissue samples by removal of paraffin with xylene and ethanol washes. Protein was degraded by proteinase K treatment (30 mg/ml for 48 hours at 55˚C) in nucleic acid lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA, and 0.7% SDS). DNA samples were then phenol/chloroform extracted and ethanol precipitated.

Loss of Heterozygosity (LOH)

Restriction fragment length polymorphism (RFLP) studies and sequence analysis were used to conduct allele specific LOH analysis. We designed forward and reverse primers that flanked each specific sequence change (Table 1). The specified region was amplified by

polymerase chain reaction (PCR) for forty cycles (15mM $MgCl_2$ (10x PCR Buffer), 5x Q, 1 unit Taq polymerase, 10 ng DNA template, 10 mg forward and reverse primer, and dH_2O per 20 μL PCR reaction) (Qiagen). For mutations and variants altering a restriction endonuclease site, the PCR products were digested with the corresponding endonuclease, separated on a 1.5% agarose, ethidium bromide stained gel and visualized under UV light (Figure 1). For mutations and variants resulting in deletion of ≥20 base pairs (bp), the PCR products were visualized on a 1.5% agarose, ethidium bromide stained gel without further treatment. Finally, for mutations and variants not fitting either of the above criteria, the PCR products were treated with ExoSap-It (USB) and sequenced (OSUCCC Nucleic Acid Shared Resource) (Figures 2,3). Sequences of control, participant normal, and participant tumor DNA were analyzed in the forward and reverse directions.

Promoter Methylation

Mutations and variants not showing definitive LOH were analyzed for putative promoter methylation. We methylated wildtype control DNA *in vitro* at 37°C for 4 hours (10 μg DNA, 30 units SSsI (CpG methyltransferase), 200x SAM (S-adenosylmethionine), 10x NEB Buffer #2, and dH_2O per 300 μl reaction) with replenishment of the methyl donor (200x SAM and 2 units SSsI) at 2 hours (New England Bio Labs). Following methylation, the methylated control DNA was phenol/chloroform extracted and ethanol precipitated. Tumor, methylated control, and unmethylated control DNA (500 ng) were bisulfite treated (EZ DNA methylation kit, Zymo Genetics). Methylated-bisulfite treated (BST) control, unmethylated-BST control, and non-BST control DNA served as controls. Bisulfite treated samples and control DNA were PCR amplified for forty cycles using previously published primers for methylation specific PCR of *BRCA1* and

BRCA2 promoter regions[6,7]. PCR products (*BRCA1* UM – 86 bp, *BRCA1* M – 182 bp, *BRCA2* UM – 145 bp, and *BRCA2* M – 180 bp) were then visualized on a 1.5% agarose, ethidium bromide stained gel and visualized under UV light for the presence or absence of methylation.

Co-occurrence with a Deleterious Mutation

Given that homozygous *BRCA1* and *BRCA2* deleterious mutations are lethal or result in extreme phenotypes, the occurrence of a deleterious mutation *in trans* with a VUS suggests the VUS is neutral[8]. We referred to mutation reports and published data to determine if VUS had been observed in co-occurrence with a known deleterious mutation. The odds of two *BRCA1* and *BRCA2* deleterious mutations occurring *in trans* are 0.0001 and 0.001, respectively[3].

Evolutionary Sequence Conservation

Missense changes of possible functional relevance were analyzed with alignment-Grantham variation, Grantham deviation (A-GVGD) to quantify their significance (http://agvgd.iarc.fr/alignments.php)[8,9]. A-GVGD places each missense mutation into a probability class based on evolutionary conservation and properties of the amino acid. Class 0 variants are expected to be neutral, and Class 65 variants are expected to be vital to protein function. Using data from Myriad Genetic Laboratories, published literature[10], and Dr. Toland's personal communication with S. Tavtiglian, we calculated odds of being deleterious for each A-GVGD class (Table 3).

Splice Site Analysis

Lymphoblastoid cell lines were established from a subset of individuals with splicing mutations or variants. RNA was isolated (RNeasy Mini Kit, Qiagen) from the cell lines and reverse transcribed (1 μg) into cDNA (iScript^{TM}, BioRad). We amplified the cDNA with primer sets on the two flanking exons and a third primer set on the causal exon. We accessed atypical splicing by comparing RT-PCR product of the tumor cell line and wildtype controls. We determined abnormal splicing when bands of the tumor sample differed from bands visualized in eleven wildtype cDNA controls. We verified abnormal splicing with sequencing.

Identification of Study Criteria for Models

Under the assumption that *BRCA1* and *BRCA2* mutation positive breast and ovarian tumors are characteristically distinct from *BRCA1* and *BRCA2* mutation negative tumors, we identified tumor characteristics to differentiate deleterious and neutral *BRCA* variants from published literature (Table 2)[3,13-51]. We specified our list to characteristics that are routinely available from pathology and mutation reports in order to make our models more applicable to a wide range of individuals. Additionally, we formed four models, one for each gene and tumor type, because the characteristics for classifying VUS varied between breast and ovarian tumors and between *BRCA1* and *BRCA2*. From the frequencies of tumor characteristics and experimental methods, we created likelihood odds for our models (Table 3). Then, we collected histopathological and clinical data from medical records and mutation reports from all of our samples for our targeted characteristics (Tables 4 and 5).

Statistical Analysis

In order to calculate the likelihood of pathogenicity for each variant, we used a modified multifactorial approach that combined the odds of causation of each independent variable[3,10-12]. The likelihood odds for each variable were taken from published odds or derived from frequencies reported in published literature (Table 2), with the exception of truncating mutations and splice site mutations. We determined odds of 1000:1 in favor of deleterious if splicing defects or truncating mutations were found and odds of 1:100 if splicing defects were not found. The likelihood odds for each independent variable (Table 3) were combined to formulate the overall odds of pathogenicity. For our ultimate classification, we followed the standard of previous work and applied the cut-off of 1000:1 in favor of deleterious and 100:1 in favor of neutral[3,10].

To calculate sensitivity we compared our results with known or previously predicted deleterious variants, and to calculate specificity we compared our results with previously predicted neutral variants.

Finally, we computed odds with and without LOH because the frequency of LOH in *BRCA* neutral variants is still uncertain and LOH data may not be easily attainable in all assessments of VUS. Thus, we categorized variants as suspected neutral or suspected deleterious if the final odds were only significant with LOH data included.

Results

Loss of Heterozygosity (LOH)

LOH occurs more frequently in *BRCA* mutation positive tumors than *BRCA* mutation negative tumors (Table 2), and thus, the presence of *BRCA1* or *BRCA2* LOH has previously been used to assess the pathogenicity of VUS[3,20,21,23,52].

For our known deleterious variants, we observed LOH of the *BRCA1* wildtype allele in 85% (28/33) of breast tumors, 100% (3/3) of ovarian tumors, and LOH of the *BRCA1* mutant allele in 3% (1/33) of breast tumors (Tables 4 and 5). We observed LOH of the *BRCA2* wildtype allele in 63% (10/16) of breast tumors, 25% (1/4) of ovarian tumors, and LOH of the *BRCA2* mutant allele in 6% (1/16) of breast tumors (Tables 4 and 5). The *BRCA1* frequencies follow closely with published rates, but the *BRCA2* frequencies are slightly lower than other published rates[3]. The observed frequency of LOH of the mutant allele was also higher than expected because published literature proposes an approximate 1% frequency of LOH of the mutant allele for deleterious mutations[3,20,21,23,52].

For our *BRCA1* VUS, we observed LOH of the wildtype allele in 32% (7/22) of tumors, LOH of the variant allele in 14% (3/22), and 55% (12/22) showed no imbalance. For *BRCA2* VUS, we observed LOH of the wildtype allele in 26% (9/35) of tumors, LOH of the variant allele in 23% (8/35), and 51% (18/35) showed no imbalance (Tables 4 and 5).

Promoter Methylation

Promoter Methylation is believed to be another mechanism that can inactivate tumor-suppressor genes[53,54]. In the absence of LOH, we hypothesized that we would detect promoter methylation in a subset of tumors. Our results, however, did not support this hypothesis because

only 3/13 *BRCA1* variants (23%) and 0/20 *BRCA2* variants (0%) showed possible methylation. Of the three *BRCA1* variants showing possible methylation, the bands seen for unmethylated DNA were much stronger than the bands showing methylation (Examples of three unmethylated *BRCA1* variants in Figure 4). Our results more clearly indicate, however, that promoter methylation plays a very small role in inactivation of *BRCA2* (Examples of *BRCA2* methylation analysis Figures 5,6). Because of the low frequencies and non-definitive results, we did not include promoter hypermethylation in our predictive models.

Co-occurrence with a Deleterious Mutation

Through mutation reports and published literature[3], we discovered three *BRCA1* (V772A, Y856H, and P334L) and two *BRCA2* (S384F and D1420Y) known neutral variants or VUS that were found to co-occur *in trans* with a known deleterious variant (Tables 4-7). Subsequently, our predictive models classified all five variants as neutral.

Evolutionary Sequence Conservation

We used A-GVGD, to quantify the pathogenicity of missense mutations in *BRCA1* and *BRCA2* using our calculated odds for each A-GVGD class (Table 3). For our *BRCA1* missense substitutions, A-GVGD categorized 1/16 (6%) as C65; 1/16 (6%) as C55, C45, or C35; 0/16 (0%) as C25 or C15; and 14/16 (88%) as C0. For our *BRCA2* missense substitutions, A-GVGD categorized 0/33 (0%) as C65; 0/33 (0%) as C55, C45, or C35; 6/33 (18%) as C25 or C15; and 27/33 (82%) as C0. Thus, all missense substitutions, except *BRCA1* C61G and L1764P, were placed in A-GVGD classes that favor neutrality. Also, C61G was previously classified as deleterious, in addition to our overall deleterious classification.

Splice Site Analysis

From a subset of individuals with intronic sequence changes, we received lymphoblastoid cell lines. We conducted splice site analysis on four different cell lines with splicing VUS and one cell line with a known deleterious splice mutation. With eleven controls, none of the splicing VUS (*BRCA1*: IVS2-14C>T and IVS20-14C>G; *BRCA2*: IVS8-12delTA and IVS23+9C>T) showed splicing defects, but the known deleterious splice mutation (*BRCA1*: IVS5-11G>T) showed a splice defect (Figures 7,8). From published literature[55] and ESEfinder, we determined that two additional *BRCA1* intronic sequence changes (IVS4-1G>T and IVS13+1G>A) showed splicing defects.

Classification

We created four models using allele specific LOH, co-occurrence with a known deleterious mutation *in trans*, sequence conservation or splice site analysis, pathological data (estrogen receptor, progesterone receptor, and Her2Neu status; tumor grade; and tumor histology), and personal cancer history (age of onset and cancer type) to classify *BRCA* sequence changes (Tables 6,7). We assessed 57 tumors with 44 known *BRCA* mutations and 56 tumors with 54 known *BRCA* neutral mutations or VUS using these four models. When we had multiple tumor samples for the same variant, we combined independent variables for classification. We classified 43/44 known deleterious mutations correctly (98% sensitivity), and we classified 16/21 mutations neutral that others have also predicted to be neutral (76% specificity)[3,8,10,12,55-60]. Of our VUS, 21/33 (64%) were classified as neutral.

Discussion

We constructed four predictive models using allele specific LOH, co-occurrence with a known deleterious mutation *in trans*, sequence conservation or splice site analysis, pathological data (estrogen receptor, progesterone receptor, and Her2Neu status; tumor grade; and tumor histology), and personal cancer history (age of onset and cancer type) to classify *BRCA* sequence changes (Tables 4-7). We assessed 57 tumors with 44 known *BRCA* mutations and 56 tumors with 54 known *BRCA* neutral mutations or VUS using these four models. We classified 43/44 known deleterious mutations correctly (98% sensitivity), and we classified 16/21 mutations neutral that others have also predicted to be neutral (76% specificity). The remainder of known mutations were classified as uncertain; no known mutations were incorrectly predicted to be deleterious or neutral. Of our VUS, 21/33 (64%) were classified as neutral (Table 8) with our four predictive models.

Our experimental studies also included the analysis of hypermethylation in the promoter regions of *BRCA1* and *BRCA2*. Our findings, however, were not definitively clear. For *BRCA1*, 3/13 variants (23%) showed possible promoter hypermethylation, but the conclusions are not definitive as the bands seen for unmethylated DNA were much stronger than the bands showing methylation. Thus, it is possible that these three variants had partial methylation in their promoter region, which may or may not inactivate *BRCA1*. For *BRCA2*, we are more confident that 0/20 variants (0%) showed methylation, and promoter hypermethylation plays a minor role in *BRCA2* inactivation. We omitted methylation data from the models in order to maintain our confidence in the models.

Additionally, our predictive models allow the assessment of pathogenicity based on characteristics, with the exception of LOH, routinely found in pathology reports, mutation

reports, published literature, and online. Our models allow characterization of VUS independent of familial information, which is often unavailable. In the future, similarly fashioned predictive models can be applied beyond *BRCA1* and *BRCA2* to further understand other disease predisposing genes.

Finally, the classification of VUS as neutral or deleterious will advance patient care by improving the value of genetic testing. More accurate genetic testing results can be used to confirm diagnosis and assess prognosis, screening options, preventative measures, and familial risk.

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Table 1: Variant Specific Primer Sequences and Annealing Temperatures

Gene	Sequence Change	Forward Primer (5' > 3')	Reverse Primer (5' > 3')	Annealing Temp. (˚C)
BRCA1	1135insA	GCC AGC TCA TTA CAG CAT GAG AAC	TCT CTG AGC ATG GCA GTT TCT G	54
BRCA1	1240delC	TGT AAT GAT AGG CGG ACT CCC A	CCC ATC ATG TGA GTC ATC AGA ACC	56
BRCA1	1294del40	AGC AGA AAC TGC CAT GCT CAG AGA	TTC ATC TAC CTC ATT TAG AAC GTG CA	56
BRCA1	1547del10	GAG GTA GAT GAA TAT TCT GGT TCT TCA GAG	GGG ACG CTC TTG TAT TAT CTG TGG	56
BRCA1	1623del5	AAC CTA TCG GAA GAA GGC AAG C	CTC CGT TTG GTT AGT TCC CTG A	56
BRCA1	1675delA	AAC CTA TCG GAA GAA GGC AAG C	CAT CAC TTG ACC ATT CTG CTC C	56
BRCA1	1793delA	TCA GGG AAC TAA CCA AAC GG	CTT TTT AGG TGC TTT TGA ATT GT	60
BRCA1	187delAG	TGT GTT AAA GTT CAT TGG AAC A	CAT AGG AAT CCC AAA TTA ATA CA	56
BRCA1	262delT	GCT CTT AAG GGC AGT TGT GAG A	TGG TTG CTT CCA ACC TAG CAT C	56
BRCA1	2530delAG	CCA AAG ATC TCA TGT TAA GTG GAG AAA GGG	GGA ACA ACC ATG AAT TAG TCC CTT GG	56
BRCA1	2553delC	CCT GGT ACT GAT TAT GGC ACT CAG GA	CTA TGC TTG TTT CCC GAC TGT GGT	58
BRCA1	2576delC	CTG GTA CTG ATT ATG GCA CTC AGG	CTA TGC TTG TTT CCC GAC TGT G	56
BRCA1	2800delAA	CCA CAG TCG GGA AAC AAG CAT AGA	CAC AGG AAA GCC TGC AGT GAT A	56
BRCA1	3118delA	CAT CTC AGT TCA GAG GCA ACG A	GCC CAC TTC ATT AGT ACT GGA ACC	56
BRCA1	3600del11	TTT CAG ATA ACT TAG AAC AGC CTA TGG GA	ATG GGT GAA AGG GCT AGG ACT C	56
BRCA1	5382insC	AGT CAG AGG AGA TGT GGT CAA TGG	GTG GTT GGG ATG GAA GAG TGA A	56
BRCA1	E143X	CCA AAG TAT GGG CTA CAG AAA CCG	GTG CCT GTA ATC CCA GCT ACT AAG	62
BRCA1	Q563X	TAC ATC AGG CCT TCA TCC TGA G	GAA GAC TTC CTC CTC AGC CTA TTC	56
BRCA1	S868X	GAC ACA GAA GGC TTT AAG TAT CCA TT	TTC TTT AAG GAC CCA GAG TGG GCA	58
BRCA1	Q1408X	GCT AGA ACT TGT AGT TCC ATA CTA GGT	GAT GGA AGG GTA GCT GTT AGA AGG	56
BRCA1	C61G	ATG GCT CTT AAG GGC AGT TGT G	GTG GTT GCT TCC AAC CTA GCA T	56
BRCA1	IVS4-1 G>T	GCT CTT AAG GGC AGT TGT GAG A	CTG TGG TTG CTT CCA ACC TAG CAT	56
BRCA1	IVS13+1 G>A	CCT TCT AAC AGC TAC CCT TCC ATC	GGC TCC ATA ATT ACC CAT GTG CTG	58
BRCA1	IVS15+1 G>A	GGG AGT CTT CAG AAT AGA AAC TAC CCA	CCA GAA TAT CTT TAT GTA GGA TTC AGA G	56
BRCA1	IVS5-11 T>G	CTA AAT CAC TGC CAT CAC ACG G	GCA CTT GAG TGT CAT TCT TGG G	56
BRCA1	E597K	TAC ATC AGG CCT TCA TCC TGA G	GAA GAC TTC CTC CTC AGC CTA TTC	56
BRCA1	E736A	CAT GAC AGC GAT ACT TTC CCA GAG	CCT GAG TGC CAT AAT CAG TAC CAG	56
BRCA1	I1275V	TGA GGA TGA AGA GCT TCC CTG CTT	GGT GAT GTT CCT GAG ATG CCT TTG C	56
BRCA1	IVS12+10G>C	CCA GTC CTG CCA ATG AGA AGA A	CTG AAT GCA AAG GAC ACC ACA C	56
BRCA1	IVS20-14C>G	TCC CTG GGA AGT AGC AGC AGA AAT	TGT AAG ACA AAG GCT GGT GCT GGA	60
BRCA1	IVS2-14C>T	TTC TCA GTT CCT GAC ACA GCA G	GGT GTT TCC TGG GTT ATG AAG GAC	56
BRCA1	IVS2-6T>C	TTC TCA GTT CCT GAC ACA GCA G	GGT GTT TCC TGG GTT ATG AAG GAC	56
BRCA1	K1109N	GCA GAA CTA GGT AGA AAC AGA GGG	CTA ACA GGT CAT CAG GTG TCT CAG	56
BRCA1	L1764P	TCC CTG GGA AGT AGC AGC AGA AAT	TGT AAG ACA AAG GCT GGT GCT GGA	60
BRCA1	P1776H	TCC CTG GGA AGT AGC AGC AGA AAT	TGT AAG ACA AAG GCT GGT GCT GGA	60
BRCA1	P334H	GCC AGC TCA TTA CAG CAT GAG AAC	CCC ATC ATG TGA GTC ATC AGA ACC	56
BRCA1	P334L	GCC AGC TCA TTA CAG CAT GAG AAC	CCC ATC ATG TGA GTC ATC AGA ACC	56
BRCA1	S127N	GAG CAT ACA TAG GGT TTC TCT TGG T	TTC GGG TTC ACT CTG TAG AAG TC	56
BRCA1	V1247I	CCT GCT TCC AAC ACT TGT TA	TGA TGT TCC TGA GAT GCC TTT	56
BRCA1	V1804D	CTA CTT AGG AGG CTG AGA TGG AAG	CCC ATA TAG CAC AGG TAC ATG CAG	56

BRCA1	V772A	CAT GAC AGC GAT ACT TTC CCA GAG	CTT CCC TAG AGT GCT AAC TTC CAG	56
BRCA1	Y856H	GAC TAA TTC ATG GTT GTT CCA	CAC ATT CAA AAG TGA CTT TTG G	61
BRCA1	P1637L	CAA CCT CTG CAT TGA AAG TTC CC	GGA TAC ACT CAC AAA TTC TTC TGG G	56
BRCA1	T1310K	GTT GCT ACC GAG TGT CTG TCT AAG	GCC CGT TCC TCT TTC TTC ATC ATC	56
BRCA2	2041delA	AGC CAC CAC CAC ACA GAA T	GAC AGA GGT ACC TGA ATC AGC A	56
BRCA2	3036del4	GAC TTG ACT TGT GTA AAC GAA CCC	CCT AAG AGT CCT GCC CAT TTG TTC	56
BRCA2	3972del4	GCA GCA AGC AAT TTG AAG GTA CAG	AAG TGC CAG TAG TCA TTT CAA	60
BRCA2	4206ins4	GTC ATG ATT CTG TCG TTT CAA TG	GCT GAT CAG TAA ATA GCA AGT CCG	56
BRCA2	4361del4	CTG CTG CCA GTA GAA ATT CTC ATA AC	GCT TCT TGA GCT TTC GCA ACT TCC	56
BRCA2	6174delT	CGA GGC ATT GGA TGA TTC AGA G	GAG CTG GTC TGA ATG TTC GTT AC	56
BRCA2	6307insA	CGC AAG ACA AGT GTT TTC TGA A	GCT TTC CAC TTG CTG TAC TAA A	58
BRCA2	6503delTT	CTG CTT TCT CTG GAT TTA GTA CAG C	GTT TAC ACA GTG CTC TGG GTT TC	56
BRCA2	7297delCT	CAC CAT GTA GCA AAT GAG GGT CTG	CTT TGG TTG GTC TGC CTG TAG T	56
BRCA2	7990del3ins2	CAG CTG TAT ACG TAT GGC GTT TC	AAG AGA AGA AAG AGG GAT GAG GG	56
BRCA2	8765delAG	CTC AGG TGA TCC ACT AAT CTC AGC	CCT TCA TGT TCT TCA AAT TCC TCC TG	56
BRCA2	8803delC	ATC TCA GCC TCC CAA AGT TCT G	CCT TCA TGT TCT TCA AAT TCC TCC TG	62
BRCA2	9481insA	TTT CAG ATT TAC CAG CCA CGG G	GCC AAC TGG TAG CTC CAA CTA ATC	56
BRCA2	Q321X	GCT GCA AAG ACC ACA TTG GAA AGT C	TGT CAC TTC CAC TCT CAA AGG GCT	56
BRCA2	S1882X	CAC CTG CAT TTA GGA TAG CCA GTG	GTG AAT GCG TGC TAC ATT CAT CAT	56
BRCA2	Y1894X	CAC CTG CAT TTA GGA TAG CCA GTG	AAC CTT ATG TGA ATG CGT GCT AC	56
BRCA2	5270delTG	GTT TCT ATT GAG ACT GTG GTG CC	CTG GTT GAC CAT CAA ATA TTC CTT CTC	56
BRCA2	A1170V	GAA GAA TCA GGA AGT CAG TTT GAA	CAG GCC AGC AAA CTT CCG TTT A	56
BRCA2	A2351G	CAC CAT GTA GCA AAT GAG GGT CTG	CTT TGG TTG GTC TGC CTG TAG T	56
BRCA2	D1352Y	CTG CTG CCA GTA GAA ATT CTC ATA AC	CCA TGA CAT GCT TCT TGA GCT TTC	56
BRCA2	E2856A	GTG GAT GGA GAA GAC ATC ATC TGG	CTG TCC CTT GTT GCT ATT CTT TGT C	56
BRCA2	H1966Y	GAA TGT AGC ACG CAT TCA CAT AAG G	TGT GAG CTG GTC TGA ATG TTC G	56
BRCA2	I2285V	AGT GGT GTT TTA AAG TGG TCA AAA	GGA TCC ACC TGA GGT CAG AAT A	60
BRCA2	IVS13+5G>C	CCT AGG CAC AAT AAA AGA TC	CGG GAA GTG TTA ACT TCT TA	56
BRCA2	IVS23+9C>T	GAG CTA ACA TAC AGT TAG CAG CG	AGG TCC ACC TCA GAA CAA GAT G	56
BRCA2	IVS8-12delTA	CAC ACT ACT CAG GAT GAC ACA CA	CAG AGG ACT TAC CAT GAC TTG CAG	58
BRCA2	K1434I	GGA AGT TGC GAA AGC TCA AGA AG	GTC TGT TTC CTC ATA ACT TAG AAT GTC C	56
BRCA2	L2106P	CTG CTT TCT CTG GAT TTA GTA CAG C	GTT TAC ACA GTG CTC TGG GTT TC	56
BRCA2	L929S	GAC TTG ACT TGT GTA AAC GAA CCC	GAA ATT GGA CCT AAG AGT CCT GCC	56
BRCA2	M2676T	GAT ACG GAA ATT GAT AGA AGC A	GCC ACT TTT TGG GTA TCT GC	60
BRCA2	V1643A	GTT TCT ATT GAG ACT GTG GTG CC	CTG GTT GAC CAT CAA ATA TTC CTT CTC	56
BRCA2	N1878K	CTT GTG ACT AGC TCT TCA CCC T	CAT CCA ATG CCT CGT AAC AAC C	56
BRCA2	S326R	CTA TGA GAA AGG TTG TGA G	CAG CGT TTG CTT CAT GGA AA	57
BRCA2	N517S	GCC ACG TAT TTC TAG CCT ACC A	GAG TCC TCC TTC TGT GAG CAA A	56
BRCA2	N588D	GTT TGC TCA CAG AAG GAG GAC T	GAC AGA GGT ACC TGA ATC AGC A	56
BRCA2	N987I	CAC AGG TGA TAA ACA AGC AAC CC	GAA ATT GGA CCT AAG AGT CCT GCC	56
BRCA2	S1172L	CCA TAA TTT AAC ACC TAG CCA AAA GG	CCA GCA AAC TTC CGT TTA ATT TC	58
BRCA2	P1819S	GCC AGT ATT GAA GAA TGT TGA AGA TC	GTA ACA ACC TGC CAT AAT TTT CGT T	61
BRCA2	P655R	TGT TTA GGT TTA TTG CAT TCT TCT GTG	GCA TGA CAG AGA ATC AGC TTC TGG	56

BRCA2	M784V	CTG CAG CAT GTC ACC CAG TA	TCA TAA TTG TTA CCT TTG AGC TTG TCT G	56
BRCA2	R2034C	CGC AAG ACA AGT GTT TTC TGA	GCT TTC CAC TTG CTG TAC TAA	58
BRCA2	R2418G	CTA CAG GCA GAC CAA CCA AAG TCT	GTT ACA GCT GCT GCT TGA TTG GAG	56
BRCA2	R2502H	TGC CAG AGA TAT ACA GGA TAT GCG	CCA TTC CTG CAC TAA TGT GTT CAT	56
BRCA2	R2973C	GAG CAG TTA AGA GCC TTG AA	CTA ACT TTA TAC TTT ATC TGG A	58
BRCA2	D1420Y	GGA AGT TGC GAA AGC TCA AGA AG	GTC TGT TTC CTC ATA ACT TAG AAT GTC C	56
BRCA2	S1424C	GGA AGT TGC GAA AGC TCA AGA AG	GTC TGT TTC CTC ATA ACT TAG AAT GTC C	56
BRCA2	S2483N	TCT TGA ACT CCC GAC CTC AGA T	CTT TCA GAG AGA TTC GAG GCA GAG	56
BRCA2	L2721H	AGG GAT GAC ACA GCT GCA AA	CTT CAA GAG GTG TAC AGG CAT CAG	56
BRCA2	S384F	TTT CCA TGA AGC AAA CGC TG	GGT GAT TCT CTT ATT CTG AAT A	56
BRCA2	T2681R	CGG AAA TTG ATA GAA GCA GAA GAT CGG	CTA ACT GGG CCT TAA CAG CAT ACC	56
BRCA2	T3211K	ACT AAA GAC TGT ACT TCA GGG CCG	ACA GGA GCC ACA TAA CAA CCA C	58
BRCA2	Y42C	TGA TCT TTA ACT GTT CTG GGT CAC	GAG TCA GCC CTT GCT CTT TGA A	56

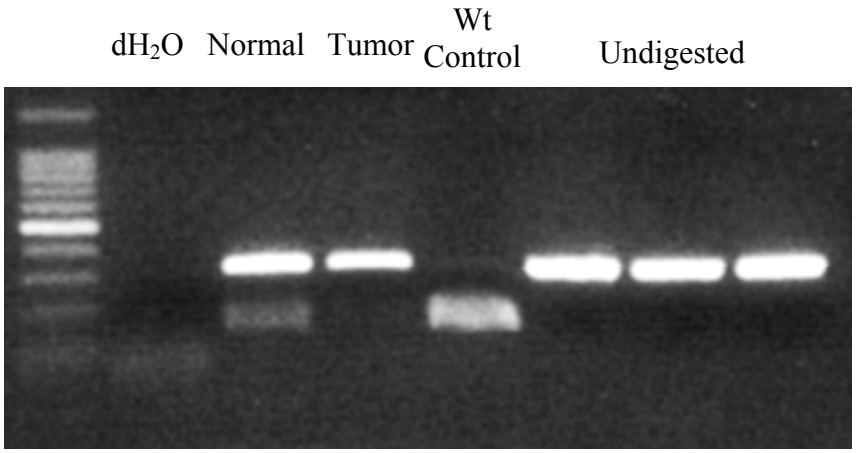


Figure 1: *BRCA1* Q563X Restriction Digest of Normal DNA (heterozygote), Tumor DNA, and Wildtype Control DNA. Hpy188I cleaves wildtype allele into 2 bands (169 and 159 bp), and is unable to cleave variant (Q563X) allele (324 bp). The Tumor DNA has a greater ratio of variant allele compared to the Normal DNA (heterozygote). Thus, there is loss of the wildtype allele.

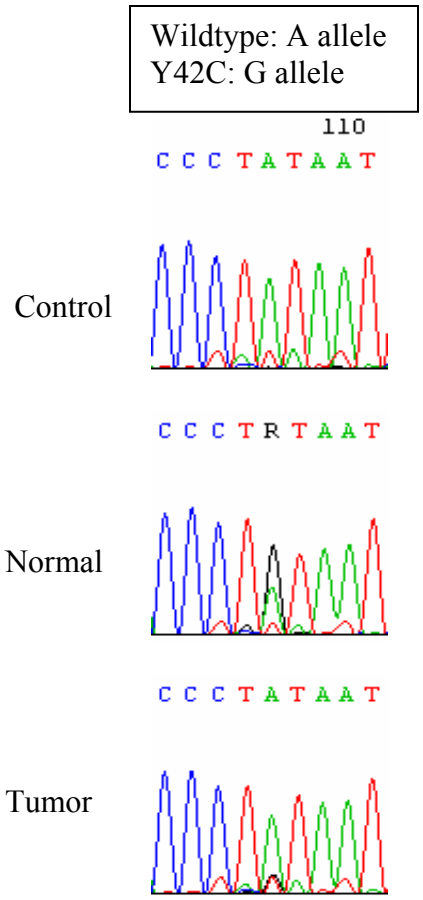


Figure 2: Sequencing of the *BRCA2* Y42C variant with Normal, Tumor, and Wildtype Control DNA. The Normal DNA has a relatively equal ratio of wildtype and variant alleles. The Tumor DNA has a greater ratio of wildtype allele compared to the Normal DNA. Thus, there is loss of the variant allele.

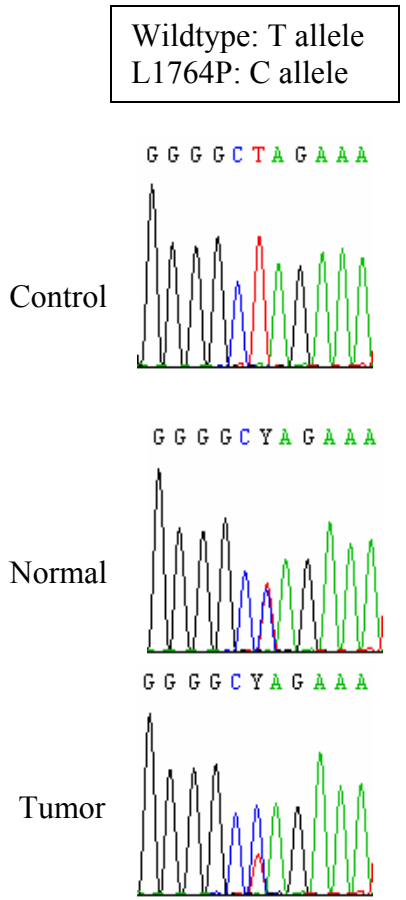


Figure 3: Sequencing of the *BRCA1* L1764P variant with Normal, Tumor, and Wildtype Control DNA. The Normal DNA has a relatively equal ratio of wildtype and variant alleles. The Tumor DNA has a greater ratio of variant allele compared to the Normal DNA. Thus, there is loss of the wildtype allele.

Table 2: Frequency of histopathological characteristics of *BRCA1*, *BRCA2* and sporadic tumors

Breast	Sporadic	*BRCA1*	*BRCA2*	Ovarian	Sporadic	*BRCA1*	*BRCA2*
BRCA1 Loss [3,16-21,23-24]	20-40% (30%)	68-100% (80%)	N/A	*BRCA1* Loss[27,29,31-34]	40-66% (40%)	72-100% (90%)	N/A
BRCA2 Loss [16,19,20,22,23,25,26]	22-50% (30%)	N/A	38-100% (70%)	*BRCA2* Loss[27,29-31,35]	30-42% (30%)	N/A	80-100% (80%)
Medullary[45-58]	1-3% (1.5%)	11-13% (12%)	N/D	Serous[29,41-44]	29-47% (34%)	40-100% (50%)	39-100% (60%)
Triple Negative[36-40,45]	10-25% (16%)	80% (80%)	N/D	Mucinous[29,41-44]	11-36% (17%)	0-3% (2%)	0-2% (1%)
ER negative [37,46,47,49-52,55,57,58]	13-37% (26%)	63-90% (83%)	N/D	Endometroid[29,41-44]	5-33% (26%)	N/A	0-29% (18%)
PR Positive[37,49,55]	58-68% (65%)	16-21% (20%)	N/D	Grade 1[43,44]	10-19% (13%)	1-3% (2%)	0-3% (3%)
Her2 positive[36,45,46, 49]	12-35% (20%)	0-3% (3%)	0-3% (3%)	Grade2[43,44]	21-34% (31%)	22%-27% (26%)	13-14% (14%)
Her2 negative [36,45,46,49,51,55,57]	70-88% (80%)	97-100% (97%)	97-100% (97%)	Grade 3[43,44]	50-55% (54%)	72-75% (72%)	81-87% (87%)
ER+/Grade 1[50,52]	27-42% (30%)	0-2.4% (2%)	5-17% (7%)	Stage 1[42,43]	24%	3-17% (17%)	0-6% (6%)
ER+/Grade 2[50,52]	11-28% (27%)	10-17% (10%)	22-45% (41%)	Stage 2[42,43]	17%	10-16% (10%)	0-6% (6%)
ER+/Grade 3[50,52]	9-17% (13%)	12-28% (13%)	28-30% (28%)	Stage 3[42,43]	40%	69-70% (69%)	82-100% (82%)
ER-/Grade 1[50,52]	3-14% (5%)	0-1% (1%)	1-4% (2%)	Stage 4[42,43]	19%	3-11% 6%	0-7% 5%
ER-/Grade 2[50,52]	12-13% (13%)	9-13% (13%)	2-17% (5%)				
ER-/Grade 3[50,52]	12-16% (16%)	62-91% (65%)	9-18% (16%)				
Grade 1 [36-38,40,45,47-50,52]	18-56% (22%)	0-9% (2.5%)	11-22% (17%)				
Grade 2 [36-38,40,45,47-50,52]	23-49% (42%)	16-26% (23%)	29-49% (43%)				
Grade 3 [36-38,40,45,47-50,52]	21-49% (36%)	66-100% (71%)	38-64% (47%)				

Ranges of frequencies reported in the literature are shown. Frequency used for weighting is in parentheses.

Abbreviations: ER, estrogen receptor; +, positive staining; - , negative staining; N/A, not applicable comparison; N/D, not calculated as the literature suggests no differences from sporadic rates

Table 3: Odds for VUS classification

Breast	*BRCA1*	*BRCA2*	Ovarian	*BRCA1*	*BRCA2*
No LOH	0.285	0.428	No LOH	0.167	0.428
Loss of wildtype	5.26	4.6	Loss of wildtype	4.45	4.6
Loss of variant[3]	0.067	0.067	Loss of variant	0.05	0.067
Medullary	8.0	N/A	Serous	1.47	1.76
ER negative	3.2	N/A	Mucinous	0.11	0.06
Triple negative	5.0	N/A	Endometroid	N/A	0.69
ER positive	0.23	N/A	Grade 1	0.15	0.23
ER negative	3.2	N/A	Grade 2	N/A	0.45
PR positive	0.31	N/A	Grade 3	1.33	1.61
PR negative	2.29	N/A	Stage 1	0.71	0.25
Her2 positive	0.15	0.15	Stage 2	0.59	0.35
Her2 negative	1.2	1.2	Stage 3	1.73	2.05
ER+/Grade 1	0.067	0.23	Stage 4	0.32	0.26
ER+/Grade 2	0.37	1.5	Diagnosis >60[10]	4.6	4.52
ER+/Grade 3	N/A	2.2	Diagnosis 50-59[10]	11.8	7.92
ER-/Grade 1	0.2	0.4	Diagnosis 40-49[10]	18.0	4.05
ER-/Grade 2	N/A	0.38	Diagnosis <40[10]	7.06	0.52
ER-/Grade 3	4.1	N/A	Unconserved domain	0.01	0.01
Grade 1	0.11	0.77	Conserved C0	0.01	0.01
Grade 2	0.55	N/A	Conserved C15,25	0.41	0.41
Grade 3	1.97	1.3	Conserved C35,45,55	1.5	1.5
Diagnosis >60[10]	1.25	1.55	Conserved C65	4.26	4.26
Diagnosis 50-59[10]	1.67	2.07	Splice defect	1000	1000
Diagnosis 40-49[10]	3.40	2.89	Not affecting splicing	0.01	0.01
Diagnosis 30-39[10]	9.65	4.97	Truncating mutation	1000	1000
Diagnosis <30[10]	15.3	4.71	*In trans* with mutation	0.0001	0.001
Unconserved domain	0.01	0.01			
Conserved C0	0.01	0.01			
Conserved C15,25	0.41	0.41			
Conserved C35,45,55	1.5	1.5			
Conserved C65	4.26	4.26			
Splice defect	1000	1000			
Not affecting splicing	0.01	0.01			
Truncating mutation	1000	1000			
In trans with mutation	0.0001	0.001			

[10]Odds for age of diagnosis as previously reported

Abbreviations: N/A, not applicable as no significant differences identified;
LOH, loss of heterozygosity; ER, estrogen receptor; +, positive; -, negative;
Bilateral, bilateral breast cancer; C, align-grantham variation, grantham deviation class.

Table 4: *BRCA1* and *BRCA2* breast tumor characteristics

Sequence Change	Sample	Conserved Domain	A-GVGD/ Class	LOH	Splice Defect	In *Trans*	Age Dx	Grade	ER	PR	Her 2	Histology
***BRCA1* truncating**												
1135insA	1463	N/A	N/A	WT	*	N/A	38	3	-	-	-	IDC
1240delC	0409	N/A	N/A	WT	*	N/A	38	2	-	-	-	DCIS
1294del40	4439	N/A	N/A	WT	*	N/A	*	4	-	-	*	IDC
1294del40	127345	N/A	N/A	WT	*	N/A	40	3	-	-	+	IDC
1294del40	78081	N/A	N/A	WT	*	N/A	51	1	-	-	*	IDC
1294del40	169184	N/A	N/A	WT	*	N/A	32	*	-	-	-	*
1547del10	21538	N/A	N/A	WT	*	N/A	33	3	-	-	+	IDC
1623del5	71759	N/A	N/A	WT	*	N/A	39	2	-	-	+	IDC
1675delA	10950	N/A	N/A	WT	*	N/A	36	3	-	-	*	IDC
1793delA	12313	N/A	N/A	WT	*	N/A	37	3	-	-	-	IDC
187delAG	45251a**	N/A	N/A	WT	*	N/A	35	3	-	-	+	IDC
187delAG	45251b**	N/A	N/A	WT	*	N/A	36	3	-	-	-	IDC
187delAG	5042	N/A	N/A	WT	*	N/A	35	3	-	-	-	IDC
2530delAG	458	N/A	N/A	WT	*	N/A	30	2	-	-	-	IDC
2553delC	4724	N/A	N/A	WT	*	N/A	27	3	-	*	*	IDC
2576delC	61933	N/A	N/A	WT	*	N/A	58	3	-	-	-	IDC
2576delC	175158	N/A	N/A	WT	*	N/A	47	*	+	+	-	IDC
2800delAA	24042a**	N/A	N/A	WT	*	N/A	39	3	*	*	*	IDC
2800delAA	24042b**	N/A	N/A	None	*	N/A	47	2	-	-	-	IDC
3118delA	11984	N/A	N/A	V	*	N/A	32	3	-	-	-	IDC
3600del11	34406	N/A	N/A	WT	*	N/A	37	2	-	-	-	Unspecified
5382insC	20758	N/A	N/A	WT	*	N/A	61	2	-	-	*	IDC
E143X	34642	N/A	N/A	WT	*	N/A	32	3	-	-	-	IDC
Q563X	61051	N/A	N/A	WT	*	N/A	47	3	+	-	-	IDC
S868X	176811	N/A	N/A	None	*	N/A	74	3	+	+	-	ILC
Q1408X	34642	N/A	N/A	None	*	N/A	29	*	-	-	-	Unspecified
***BRCA1* splice defect**												
IVS4-1 G>T	2364a**	N/A	N/A	WT	Yes[54]	No	48	3	-	-	*	DCIS, LCIS
IVS4-1 G>T	2364b**	N/A	N/A	WT	Yes[54]	No	49	1	+	+	-	DCIS, LCIS
IVS13+1 G>A	98453	N/A	N/A	None	Yes[@]	No	41	2	-	-	-	IDC
IVS15+1 G>A	2-112-140	N/A	N/A	WT	Yes[@]	No	*	4	-	-	*	IDC
IVS5-11 T>G	45949	N/A	N/A	WT	Yes	No	59	2	-	-	-	Medullary
***BRCA1* intronic**												
IVS12+10G>C	78081	N/A	N/A	None	*	No	51	1	-	-	*	IDC
IVS20-14C>G	168788	N/A	N/A	None	No	No	34	3	+	+	-	IDC
IVS2-14C>T	64703	N/A	N/A	V	No	No	44	1	+	+	-	IDC
IVS2-6T>C	15007	N/A	N/A	None	*	No	38	*	+	+	-	DCIS
***BRCA1* missense**												

C61G	14068	Yes	C65	WT	*	No	32	3	-	-	+	IDC
C61G	19882	Yes	C65	WT	*	No	29	3	-	-	-	IDC
E597K	98286	No	C0	None	*	No	45	1	+	+	*	IDC
E736A	15102	No	C0	None	*	No	44	*	+	+	-	DCIS
I1275V	4061	No	C0	WT	*	No	37	3	-	-	-	IDC
K1109N	0477a**	No	C0	None	*	No	35	*	*	*	*	DCIS
K1109N	0477b**	No	C0	None	*	No	49	2	+	+	-	IDC
L1764P	4744	Yes	C35	WT	*	No	32	3	-	-	+	IDC
P1637L	175158	Close	C0	WT	*	No	47	*	+	+	-	IDC
P1776H	154045	No	C0	WT	*	No	48	3	+	+	-	*
P1776H	133886	No	C0	WT	*	No	55	*	*	*	*	Unspecified
P334H	34402	No	C0	None	*	No	38	2	+	+	-	IDC
S127N	4060	No	C0	V	*	No	38	*	*	*	*	IDC
V1247I	11092	No	C0	None	*	No	40	3	+	+	-	IDC
V1804D	16719	Yes	C0	V	*	No	58	2	+	-	+	IDC
V1804D	73481	Yes	C0	*	*	No	59	1	+	+	*	IDC
V772A	78081	No	C0	WT	*	Yes	47	1	-	-	*	IDC
T1310K	176831	No	C0	None	*	No	45	*	*	*	*	DCIS
Y856H	1995	No	C0	None	*	Yes	45	3	+	+	+	DCIS
Y856H	4294	No	C0	None	*	Yes	47	*	*	*	*	DCIS
***BRCA2* truncating**												
2041delA	46662	N/A	N/A	None	*	N/A	34	3	+	+	-	IDC
3036del4	4208	N/A	N/A	U	*	N/A	*	2	+	+	-	IDC
3036del4	4310	N/A	N/A	WT	*	N/A	36	3	+	+	+	IDC
3036del4	542	N/A	N/A	WT	*	N/A	43	*	+	+	*	IDC
3972del4	5-077	N/A	N/A	WT	*	N/A	*	*	+	+	*	IDC
4206ins4	26949	N/A	N/A	None	*	N/A	37	1	+	+	-	LCIS
4361del4	2609	N/A	N/A	WT	*	N/A	*	*	-	+	-	IDC
5270delTG	169989	N/A	N/A	None	*	N/A	46	2	+	-	*	IDC
6174delT	4317	N/A	N/A	WT	*	N/A	46	3	-	-	-	IDC
6503delTT	35962	N/A	N/A	WT	*	N/A	46	2	+	+	+	IDC
7990del3ins2	15494	N/A	N/A	WT	*	N/A	41	3	+	-	-	IDC
8765delAG	5702	N/A	N/A	*	*	N/A	50	*	-	-	*	IDC
8803delC	80659	N/A	N/A	None	*	N/A	36	*	+	+	*	Unspecified
9481insA	94467	N/A	N/A	WT	*	N/A	64	2	+	+	+	IDC
Q321X	3-744	N/A	N/A	V	*	N/A	*	4	+	+	-	IDC
S1882X	3553	N/A	N/A	WT	*	N/A	37	3	+	+	-	IDC
Y1894X	11008	N/A	N/A	WT	*	N/A	26	*	+	+	-	IDC
***BRCA2* intronic**												
IVS13+5G>C	16004	N/A	N/A	WT	*	No	46	2	+	+	-	IDC
IVS23+9C>T	12673	N/A	N/A	WT	No	No	40	2	+	+	+	IDC
IVS8-12delTA	64703	N/A	N/A	None	No	No	44	1	+	+	-	IDC
***BRCA2* Missense**												

A1170V	143226	No	C0	*	*	No	52	3	-	+	+	IDC
A2351G	2664	No	C0	None	*	No	61	3	-	-	-	IDC
D1352Y	11193	No	C0	WT	*	No	47	2	+	+	-	IDC
E2856A	5047	Yes	C0	V	*	No	45	2	+	+	-	IDC
H1966Y	46345	No	C0	V	*	No	44	3	-	-	-	IDC
I2285V	4112	No	C25	V	*	No	*	2	+	+	+	IDC
K1434I	34415	No	C15	None	*	No	58	*	+	-	*	DCIS
L2106P	161491	No	C0	None	*	No	57	3	+	+	-	IDC
L929S	14388	No	C0	None	*	Yes	37	2	+	+	-	IDC
M2676T	565	Yes	C0	None	*	No	49	*	+	*	*	IDC
N1878K	14573a**	No	C0	V	*	No	63	1	+	-	-	IDC
N1878K	14573b**	No	C0	V	*	No	67	2	+	+	-	IDC
N517S	11859	No	C0	None	*	No	45	1	+	+	*	IDC
N588D	0772	No	C0	WT	*	No	56	2	+	+	+	IDC
N987I	14388	No	C0	None	*	Yes	37	2	+	+	-	IDC
P1819S	4146	No	C0	None	*	No	57	1	+	+	-	IDC
P655R	11631	No	C0	None	*	No	38	3	+	+	-	IDC
R2034C	2098	No	C0	None	*	Yes	75	3	-	-	+	IDC
R2418G	26664	Yes	C0	None	*	No	61	2	+	+	-	IDC
R2502H	98245	Yes	C0	None	*	No	58	3	+	+	-	IDC
R2973C	2382	Yes	C25	WT	*	No	52	2	+	+	-	IDC
S1424C	155088	No	C0	None	*	No	55	*	*	*	*	*
S2483N	25006	Yes	C0	None	*	No	48	1	+	+	-	IDC
S384F	6996	No	C0	WT	*	Yes	49	2	+	+	+	IDC
T2681R	25705	Yes	C0	WT	*	No	34	3	-	-	-	IDC
T3211K	59457	Yes	C0	None	*	No	52	3	-	-	-	*
Y42C	4620	No	C0	V	*	No	40	*	-	-	-	IDC

[@]Change predicted to affect splicing by splice finder

[54]Change shown to affect splicing by others

[**]Individual has bilateral breast cancer and both tumors were included

Abbreviations: *, No data; N/A, not applicable; WT, loss of wild type allele; V, loss of variant allele; U, uncertain loss; +, positive; -, negative; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma in situ; LCIS, lobular carcinoma in situ

Table 5: Ovarian tumor characteristics

Sequence Change	Sample	Conserved domain	LOH	A- GVGD Class	In *Trans*	Stage	Grade	Age	Histology
***BRCA1* Truncating**									
1135insA	24127	N/A	WT	N/A	No	*	3C	59	Papillary Serous
262delT	0690	N/A	WT	N/A	No	*	3C	63	Endometroid
3600del11	10945	N/A	WT	N/A	No	*	3	41	Serous
***BRCA1* Missense**									
P334L	6167	No	WT	C0	Yes	*	*	39	Unspecified
***BRCA2* Truncating**									
6307insA	4945	N/A	none	N/A	N/A	*	*	*	Unspecified
7297delCT	0947	N/A	none	N/A	N/A	*	3C	22	Serous
7990del3ins2	540	N/A	none	N/A	N/A	*	*	46	Unspecified
9481insA	23722	N/A	WT	N/A	N/A	*	3C	59	Papillary Serous
***BRCA2* Missense**									
A1170V	97594	No	V	C0	No	*	*	59	*
D1420Y	0690	No	none	C15	Yes	*	3C	63	Endometrioid
L2721H	6167	Yes	WT	C25	No	*	*	39	Unspecified
M784V	11073	No	V	C0	No	3C	3	45	Unspecified
S1172L	5701	No	none	C15	No	3C	*	58	Serous
S326R	5701	No	none	C0	Yes	3C	*	58	Serous
V1643A	76049	No	WT	C0	No	PT3C	*	50	Serous

Abbreviations: N/A, not applicable; *, no data; LOH, loss of heterozygosity; WT, loss of wildtype allele; V, loss of variant or mutant allele

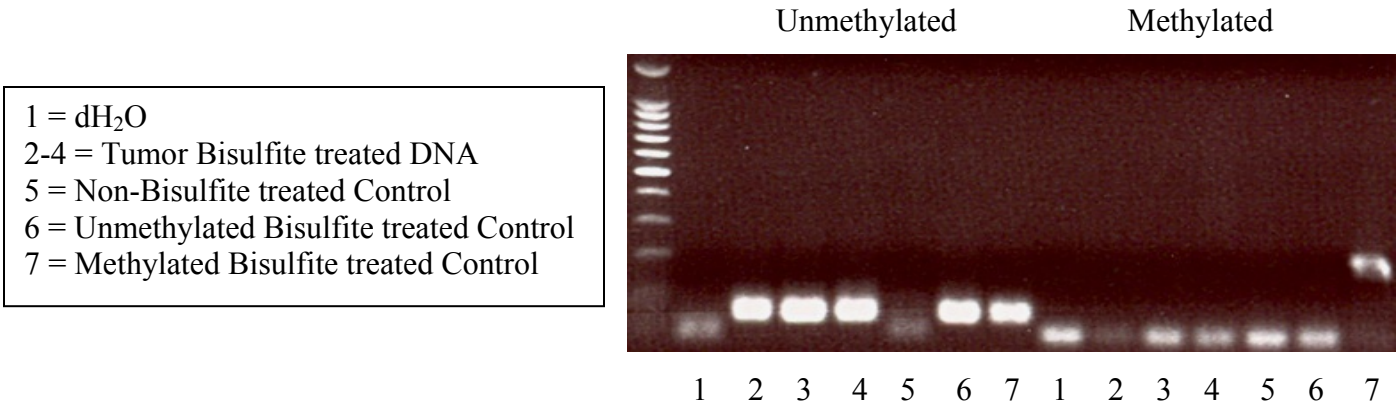
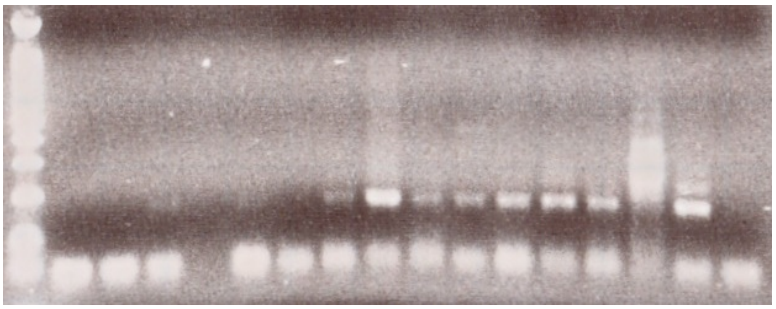


Figure 4: *BRCA1* Unmethylated and Methylated PCR Products. The primers for unmethylated DNA yielded bands (86 bp) for all samples except the negative controls of dH_2O and Non-Bisulfite treated Control DNA. The primers for methylated DNA yielded a band (182 bp) only for the Methylated Bisulfite treated Control DNA.

Unmethylated

1 = dH_2O
2-13 = Tumor Bisulfite treated DNA
14 = Non-Bisulfite treated Control
15 = Unmethylated Bisulfite treated Control
16 = Methylated Bisulfite treated Control

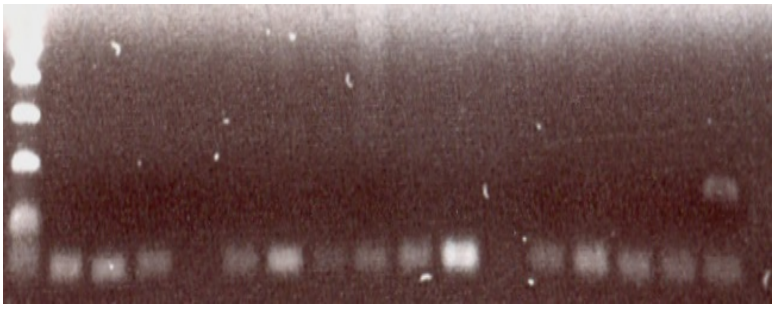


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 5: *BRCA2* Unmethylated PCR Products. The primers for unmethylated DNA yielded bands (145 bp) for nine samples of Tumor Bisulfite treated DNA and the Unmethylated Bisulfite treated Control DNA. Bands were absent for dH_2O, three samples of Tumor Bisulfite treated DNA, Non-Bisulfite treated Control DNA, and the Methylated Bisulfite treated Control DNA.

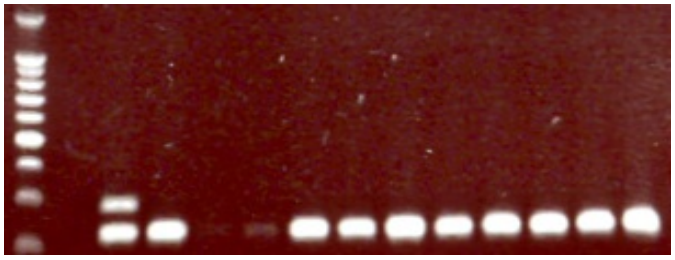
Methylated

1 = dH_2O
2-13 = Tumor Bisulfite treated DNA
14 = Non-Bisulfite treated Control
15 = Unmethylated Bisulfite treated Control
16 = Methylated Bisulfite treated Control



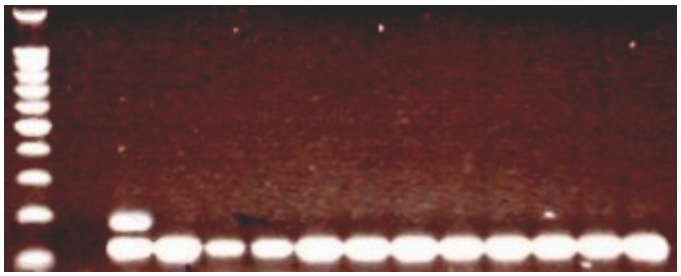
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 6: *BRCA2* Methylated PCR Products. The primers for methylated DNA yielded a band (180 bp) only for the Methylated Bisulfite treated Control DNA. Bands were absent for all other samples.



Tumor cDNA Wildtype Controls

Figure 7: Splice Site Analysis of IVS5-11G>T. The RT-PCR Products were amplified with primers that annealed to the two flanking exons (exons 5 and 7). Aberrant splicing was observed in the Tumor cDNA as it produced two bands, which differed from the one band (232 bp) produced in eleven other wildtype cDNA controls.



Tumor cDNA Wildtype Controls

Figure 8: Splice Site Analysis of IVS5-11G>T. The RT-PCR Products were amplified with primers that annealed to one flanking exon (exon 5) and the causal exon (exon 6). Aberrant splicing was observed in the Tumor cDNA as it produced two bands, which differed from the one band (132 bp) produced in eleven other wildtype cDNA controls.

Table 6: Prediction of deleterious status for *BRCA1* and *BRCA2* breast tumors

Sequence Change	Sample	LOH	A-GVGD/ Mutation	Splice	In *Trans*	Age	Triple Neg	ER	PR	HER	Grade ER	Grade	HP	Odds	Odds no LOH	Int
***BRCA1* Truncating**																
1135insA[#]	1463	5.26	1000	1	1	9.65	5	1	1	1	1	1.97	1	$4.9x10^5$	$9.5x10^4$	D
1240delC	0409	5.26	1000	1	1	9.65	5	1	1	1	1	0.55	1	$1.4x10^5$	$2.6x10^4$	D
1294del40	4439	5.26	1000	1	1	1	1	1	2.29	1	4.1	1	1	$4.9x10^4$	9389	D
1294del40	127345	5.26	1000	1	1	3.4	1	1	2.29	0.15	4.1	1	1	$2.5x10^4$	4788	D
1294del40	78081[+]	5.26	1000	1	1	1.67	1	1	2.29	1	0.2	1	1	4023	765	DS
1294del40	169184	5.26	1000	1	1	9.65	5	1	1	1	1	1	1	$2.5x10^5$	$4.8x10^4$	D
1294del40	Combo	765.5	1000	1	1	9.65	5	1	11.53	0.15	3.362	1	1	$2.1x10^8$	$2.8x10^5$	D
1547del10	21538	5.26	1000	1	1	9.65	1	1	2.29	0.15	4.1	1	1	$7.1x10^4$	$1.4x10^4$	D
1623del5	71759	5.26	1000	1	1	9.65	1	3.2	2.29	0.15	1	1	1	$3.1x10^4$	5834	D
1675delA	10950	5.26	1000	1	1	9.65	1	1	2.29	1	4.1	1	1	$4.8x10^5$	$9x10^4$	D
1793delA	12313	5.26	1000	1	1	9.65	5	1	1	1	1	1.97	1	$4.9x10^5$	$9.5x10^4$	D
187delAG	45251a*	5.26	1000	1	1	9.65	1	1	2.29	0.15	4.1	1	1	$7.1x10^4$	$1.4x10^4$	D
187delAG	45251b*	5.26	1000	1	1	9.65	5	1	1	1	1	1.97	1	$4.9x10^5$	$9.5x10^4$	D
187delAG	5042	5.26	1000	1	1	9.65	5	1	1	1	1	1.97	1	$4.9x10^5$	$9.5x10^4$	D
187delAG	Combo	145.53	1000	1	1	9.65	25	1	2.29	0.15	4.1	3.88	1	$1.9x10^8$	$1.3x10^6$	D
2530delAG	458	5.26	1000	1	1	9.65	5	1	1	1	1	0.55	1	$1.4x10^5$	$2.7x10^4$	D
2553delC	4724	5.26	1000	1	1	15.3	1	1	1	1	4.1	1	1	$3.3x10^5$	$6.3x10^4$	D
2576delC	61933	5.26	1000	1	1	1.67	5	1	1	1	1	1.97	1	$8.7x10^4$	$1.6x10^4$	D
2576delC	175158	5.26	1000	1	1	3.4	1	0.23	0.31	1.2	1	1	1	1530	291	DS
2576delC	Combo	27.66	1000	1	1	3.4	5	0.23	0.31	1.2	1	1.97	1	$7.9x10^4$	2865	D
2800delAA	24042a*	5.26	1000	1	1	9.65	1	1	1	1	1	1.97	1	$9.9x10^4$	$1.9x10^4$	D
2800delAA	24042b*	0.285	1000	1	1	3.4	5	1	1	1	1	0.55	1	2665	9350	D
2800delAA	Combo	1.499	1000	1	1	9.65	5	1	1	1	1	1.08	1	$7.8x10^4$	$5.2x10^4$	D
3118delA	11984	0.0667	1000	1	1	9.65	5	1	1	1	1	1.97	1	6340	$9.5x10^4$	D
3600del11	34406	5.26	1000	1	1	9.65	5	1	1	1	1	0.55	1	$1.4x10^5$	$2.7x10^4$	D
5382insC	20758	5.26	1000	1	1	1.25	1	1	2.29	1	4.1	1	1	$6.2x10^4$	$1.2x10^4$	D
E143X	34642	5.26	1000	1	1	9.65	5	1	1	1	1	1.97	1	$4.9x10^5$	$9.5x10^4$	D
Q563X	61051	5.26	1000	1	1	3.4	1	0.23	2.29	1.2	1	1.97	1	$2.2x10^4$	4233	D
S868X	176811	0.285	1000	1	1	1.25	1	0.23	0.31	1.2	1	1.97	1	60	210	U
Q1408X	34642	0.285	1000	1	1	15.3	5	1	1	1	1	1	1	$4.9x10^4$	$1.8x10^5$	D
***BRCA1* Splicing**																
IVS4-1 G>T	2364a*	5.26	1	1000	1	3.4	1	1	2.29	1	4.1	1	1	$1.7x10^5$	$3.2x10^4$	D
IVS4-1 G>T	2364b*	5.26	1	1000	1	3.4	1	0.23	0.31	1.2	1	0.11	1	168	32	U
IVS4-1 G>T	Combo	27.66	1	1000	1	3.4	1	0.23	0.71	1.2	4.1	0.11	1	8314	300	DS [55]
IVS13+1 G>A	98453	0.285	1	1000	1	3.4	5	1	1	1	1	0.55	1	2665	9350	D[10]
IVS15+1 G>A	2-112-140	5.26	1	1000	1	1	1	1	2.29	1	4.1	1	1	$4.9x10^4$	9389	D[10]

IVS5-11 T>G	45949	5.26	1	1000	1	1.67	5	1	1	1	1	0.55	4.3	$1x10^{5}$	$1.9x10^{4}$	D[56]
***BRCA1* intronic**																
IVS12+10G>C	78081[+]	0.285	1	1	1	1.67	1	1	2.29	1	0.2	1	1	0.218	0.765	U
IVS20-14C>G	168788	0.285	1	0.01	1	9.65	1	0.23	0.31	1.2	1	1.97	1	0.0045	0.0162	NS
IVS2-14C>T	64703	0.0667	1	0.01	1	3.4	1	1	0.31	1.2	1	0.11	1	0.0009	0.001	N
IVS2-6T>C	15007	0.285	1	1	1	9.65	1	0.23	0.31	1.2	1	1	1	0.235	0.826	U
***BRCA1* missense**																
C61G	14068	5.26	4.26	1	1	9.65	1	1	2.29	0.15	4.1	1	1	304	58	U
C61G	19882	5.26	4.26	1	1	15.3	5	1	1	1	1	1.97	1	3377	642	DS
C61G	Combo	27.66	4.26	1	1	15.3	5	1	2.29	0.15	4.1	1.97	1	$2.5x10^{4}$	904	DS
E597K	98286	0.285	0.01	1	1	3.4	1	0.23	0.31	1	1	0.11	1	$7.6x10^{-5}$	0.00027	N[10]
E736A	15102	0.285	0.01	1	1	3.4	1	0.23	0.31	1.2	1	1	1	0.00008	0.0029	N
I1275V	4061	5.26	0.01	1	1	9.65	5	1	1	1	1	1.97	1	4.99	0.95	U[10]
K1109N	0477a*	0.285	0.01	1	1	9.65	1	1	1	1	1	1	1	0.028	0.097	U
K1109N	0477b*	1	0.01	1	1	3.4	1	1	2.29	1.2	0.37	1	1	0.092	0.035	U
K1109N	Combo	0.285	0.01	1	1	9.65	1	1	2.29	1.2	0.37	1	1	0.074	0.098	U[10]
L1764P	4744	5.26	1.5	1	1	9.65	1	1	2.29	0.15	4.1	1	1	107	20.4	U[10,59]
P1637L	175158	5.26	0.01	1	1	3.4	1	0.23	0.31	1.2	1	1	1	0.015	0.0029	N
P1776H	154045	5.26	0.01	1	1	3.4	1	0.23	0.31	1.2	1	1.97	1	0.030	0.057	U
P1776H	133886	5.26	0.01	1	1	1.67	1	1	1	1	1	1	1	0.088	0.017	U
P1776H	Combo	145.5	0.01	1	1	3.4	1	0.23	0.31	1.2	1	1.97	1	0.159	0.006	N
P334H	34402	0.285	0.01	1	1	9.65	1	1	0.31	1.2	0.37	1	1	0.003	0.01	N
S127N	4060	0.0667	0.01	1	1	9.65	1	1	1	1	1	1	1	0.0064	0.097	NS
T1310K	176831	0.286	0.01	1	1	3.4	1	1	1	1	1	1	1	0.0097	0.034	NS
V1247I	11092	0.285	0.01	1	1	3.4	1	0.23	0.31	1.2	1	1.97	1	0.0016	0.0057	N[10]
V1804D	16719	0.0667	0.01	1	1	1.67	1	1	2.29	0.15	0.37	1	1	0.00014	0.014	NS
V1804D	73481	1	0.01	1	1	1.67	1	1	0.31	1	0.067	1	1	0.00034	0.00034	N
V1804D	Combo	0.0667	0.01	1	1	1.67	1	1	0.71	0.15	0.025	1	1	$2.9x10^{-6}$	$4.4x10^{-5}$	N[10]
V772A	78081[+]	5.26	0.01	1	0.0001	3.4	1	1	2.29	1	0.2	1	1	$8.2x10^{-6}$	$1.6x10^{-6}$	N[8]
Y856H	1995	0.285	0.01	1	0.0001	3.4	1	0.23	0.31	0.15	1	1.97	1	$2x10^{-8}$	$7x10^{-8}$	N
Y856H	4294	0.285	0.01	1	0.0001	3.4	1	1	1	1	1	1	1	$9x10^{-7}$	$3x10^{-6}$	N
Y856H	Combo	0.08	0.01	1	0.0001	1	1	0.23	0.31	0.15	1	1.97	1	$5.7x10^{-9}$	$7x10^{-8}$	N[8,56]
BRCA2 truncating																
2041delA	46662	0.428	1000	1	1	4.97	1	1	1	1.2	2.2	1	1	5616	$1.3x10^{4}$	D
3036del4	4208	1	1000	1	1	1	1	1	1	1.2	1.5	1	1	1800	1800	D
3036del4	4310	4.6	1000	1	1	4.97	1	1	1	0.15	2.2	1	1	7544	1640	D
3036del4	542	4.6	1000	1	1	2.89	1	1	1	1	1	1	1	$1.3x10^{4}$	2890	D
3036del4	Combo	21.16	1000	1	1	2.89	1	1	1	0.18	3.3	1	1	$3.6x10^{4}$	1717	D
3972del4	5-077	4.6	1000	1	1	1	1	1	1	1	1	1	1	4600	1000	D
4206ins4	26949	0.428	1000	1	1	4.97	1	1	1	1.2	0.23	1	1	587	1371	D
4361del4	2609	4.6	1000	1	1	1	1	1	1	1.2	1	1	1	5520	1200	D

5270delTG	169989	0.428	1000	1	1	2.89	1	1	1	1	1.5	1	1	1855	4335	D
6174delT	4317	4.6	1000	1	1	2.89	1	1	1	1.2	1	1.3	1	$2.1x10^{4}$	4508	D
6503delTT	35962	4.6	1000	1	1	2.89	1	1	1	0.15	1.5	1	1	2991	650	DS
7990del3ins2[#]	15494	4.6	1000	1	1	2.89	1	1	1	1.2	2.2	1	1	$3.5x10^{4}$	7630	D
8765delAG	5702	0.428	1000	1	1	2.07	1	1	1	1	1	1	1	886	2070	D
8803delC	80659	0.428	1000	1	1	4.97	1	1	1	1	1	1	1	2127	4970	D
9481insA	94467	4.6	1000	1	1	1.55	1	1	1	0.15	1.5	1	1	1604	349	DS
Q321X	3-744	0.0667	1000	1	1	1	1	1	1	1.2	2.2	1	1	176	2640	D
S1882X	3553	4.6	1000	1	1	4.97	1	1	1	1.2	2.2	1	1	$6x10^{4}$	$1.3x10^{4}$	D
Y1894X	11008	4.6	1000	1	1	4.97	1	1	1	1.2	1	1	1	$2.6x10^{4}$	5652	D
***BRCA2* Intronic**																
IVS13+5G>C	16004	4.6	1	1	1	2.89	1	1	1	1.2	1.5	1	1	23.93	5.2	U
IVS23+9C>T	12673	4.6	1	0.01	1	2.89	1	1	1	0.15	1.5	1	1	0.03	0.0065	N
IVS8-12delTA	64703	0.428	1	0.01	1	2.89	1	1	1	1.2	0.23	1	1	0.003	0.008	N
***BRCA2* Missense**																
A1170V[#]	143226	1	0.01	1	1	2.07	1	1	1	0.15	1	1.3	1	0.027	0.027	U
A2351G	2664	0.428	0.01	1	1	1.55	1	1	1	1.2	1	1.3	1	0.010	0.024	NS
D1352Y	11193	4.6	0.01	1	1	2.89	1	1	1	1.2	1.5	1	1	0.239	0.05	U
E2856A	5047	0.0667	0.01	1	1	2.89	1	1	1	1.2	1.5	1	1	0.0034	0.52	NS[3]
H1966Y	46345	0.0667	0.01	1	1	2.89	1	1	1	1.2	1	1.3	1	0.003	0.045	NS
I2285V	4112	0.0667	0.01	1	1	1	1	1	1	0.15	1.5	1	1	0.001	0.002	N[10]
K1434I	34415	0.428	0.01	1	1	2.07	1	1	1	1	1	1	1	0.009	0.027	NS
L2106P	161491	0.428	0.01	1	1	2.07	1	1	1	1.2	2.2	1	1	0.023	0.054	U
L929S	14388**	0.428	0.01	1	0.001	4.97	1	1	1	1.2	1.5	1	1	$3.8x10^{-5}$	$8.9x10^{-5}$	N[60]
M2676T	565	0.428	0.01	1	1	1	1	1	1	1	1	1	1	0.012	0.029	U
N1878K	14573a*	0.0667	0.01	1	1	1.55	1	1	1	1.2	0.23	1	1	0.0003	0.004	N
N1878K	14573b*	0.0667	0.01	1	1	1.55	1	1	1	1.2	1.5	1	1	0.002	0.028	NS
N1878K	Combo	0.0045	0.01	1	1	1.55	1	1	1	1.44	0.345	1	1	$3.4x10^{-5}$	0.008	N
N517S	11859	0.428	0.01	1	1	2.89	1	1	1	1	0.23	1	1	0.003	0.007	N
N588D	0772	4.6	0.01	1	1	2.07	1	1	1	0.15	1.5	1	1	0.02	0.005	N
N987I	14388**	0.428	0.01	1	0.001	4.97	1	1	1	1.2	1.5	1	1	$3.8x10^{-5}$	$8.9x10^{-5}$	N[60]
P1819S	4146	0.428	0.01	1	1	2.07	1	1	1	1.2	0.23	1	1	0.002	0.006	N[10]
P655R	11631	0.428	0.01	1	1	4.97	1	1	1	1.2	2.2	1	1	0.056	0.131	U
R2034C	2098	0.428	0.01	1	0.001	1.55	1	1	1	0.15	1	1.3	1	0.00001	$2x10^{-6}$	N[59]
R2418G	26664	0.428	0.01	1	1	1.55	1	1	1	1.2	1.5	1	1	0.011	0.028	U
R2502H	98245	0.428	0.01	1	1	2.07	1	1	1	1.2	2.2	1	1	0.023	0.054	U
R2973C	2382	4.6	0.41	1	1	2.07	1	1	1	1.2	1.5	1	1	7.03	1.53	U[10]
S1424C	155088	0.428	0.01	1	1	2.07	1	1	1	1	1	1	1	0.009	0.008	N
S2483N	25006	0.428	0.01	1	1	2.89	1	1	1	1.2	0.23	1	1	0.003	0.008	N
S384F	6996	4.6	0.01	1	0.001	2.89	1	1	1	0.15	1.5	1	1	$2.9x10^{-5}$	$6.5x10^{-6}$	N[3, 8,57]
T2681R	25705	4.6	0.01	1	1	4.97	1	1	1	1.2	1	1	1	0.274	0.059	U

T3211K	59457	0.428	0.01	1	1	2.07	1	1	1	1	1	1	1	0.009	0.021	NS
Y42C	4620	0.0667	0.01	1	1	2.89	1	1	1	1.2	1	1	1	0.002	0.035	NS [3,12]

+Individual has both *BRCA1* mutation and 2 *BRCA1* variants
*Individual has bilateral breast cancer, both tumors tested
[#]Mutation also seen in a different individual with ovarian cancer
**Individual has both *BRCA2* L929S and N987I variants
[3,8,10,12,55,56,58-60]Reclassified as neutral or deleterious by other studies
Abbreviations: Combo, odds combined for all samples with variant; Triple Neg, triple negative; ER, estrogen receptor status; PR, progesterone receptor status; Her2, Her2 Neu status; HP, histopathology; D, Deleterious; N, neutral; U, uncertain; DS, suspected deleterious; NS, suspected neutral; Int, Interpretation

Table 7: Prediction of deleterious status in *BRCA1* and *BRCA2* ovarian tumors

Sequence Change	Sample	LOH	A-GVGD/ Mutation	Splice	In *Trans*	Stage	Grade	HP	Age	Combined Odds	Odds No LOH	Interpretation
***BRCA1* Truncating**												
1135insA[+]	24127	4.45	1000	1	1	1	1.33	1.47	11.8	102662.3	23070.2	Deleterious
262delT	0690[#]	4.45	1000	1	1	1	1.33	1	4.6	27225.1	6118	Deleterious
3600del11	10945	4.45	1000	1	1	1	1.33	1.47	18	156603.51	35192	Deleterious
***BRCA1* Missense**												
P334L	6167[$]	4.45	0.01	1	0.0001	1	1	1	7.06	0.0000314	.0000071	Neutral[10]
***BRCA2* Truncating**												
6307insA	4945	0.428	1000	1	1	1	1	1	1	428	1000	Deleterious
7297delCT	0947	0.428	1000	1	1	1	1.61	1.76	0.52	630.65	1473.47	Deleterious
7990del3ins2[+]	540	0.428	1000	1	1	1	1	1	4.05	1733.4	4050	Deleterious
9481insA	23722	4.6	1000	1	1	1	1.61	1.76	7.92	103233.72	22442.11	Deleterious
***BRCA2* Missense**												
A1170V[+]	97594	0.067	0.01	1	1	1	1	1	7.92	0.00528	0.0792	Neutral Suspected
D1420Y	0690[#]	0.428	0.01	1	0.001	1	1.61	0.69	4.52	0.0000215	.00005	Neutral[59]
L2721H	6167[$]	4.6	0.41	1	1	1	1	1	0.52	0.9807	0.2132	Uncertain
M784V	11073	0.067	0.01	1	1	2.05	1.61	1	4.05	0.009	0.1337	Neutral Suspected
S1172L	5701**	0.428	0.01	1	1	2.05	1	1.76	7.92	0.1223	0.2857	Uncertain[10]
S326R	5701**	0.428	0.01	1	0.001	2.05	1	1.76	7.92	0.000122	0.00028	Neutral
V1643A	76049	4.6	0.01	1	1	2.05	1	1.76	7.92	1.3145	0.2858	Uncertain

[+]Change also seen in a different individual with breast cancer

[#]Individual carries both *BRCA1* 262delT and *BRCA2* variant D1420Y

[$]Individual carries both *BRCA1* P334L and *BRCA2* L2721H

**Individual carries both *BRCA2* S1172L and S326R

[10,59]Reclassified as neutral by other studies

Abbreviations: LOH, loss of heterozygosity, A-GVGD, align-grantham variation, grantham deviation; HP, histopathology

Table 8: Variants of Uncertain Significance Reclassified as Neutral

BRCA1	*BRCA2*
IVS2-14C>T	IVS8-12delTA
IVS20-14C>G	IVS23+9C>T
S127N	N517S
P334H	N588D
E736A	M784V
T1310K	A1170V
P1637L	S1424C
P1776H	K1434I
	N1878K
	H1966Y
	A2351G
	S2483N
	T3211K